The immune system of the freshwater zebra mussel, *Dreissena polymorpha*, decrypted by proteogenomics of hemocytes and plasma compartments.

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Highlights

- Proteogenomic analysis of the zebra mussel hemolymph
- Large-scale description of the molecular mechanisms involved in mussel immunity
- Discovery of immune proteins rarely observed in the hemolymph of bivalves

Abstract

The immune system of bivalves is of great interest it reflects the health status of these organisms during stressful conditions. While immune molecular responses are well documented for marine bivalves, few information is available for continental bivalves such as the zebra mussel, *Dreissena polymorpha*. A proteogenomic approach was conducted on both hemocytes and plasma to identified immune proteins of this non-model species. Combining transcriptomic sequences with mass spectrometry data acquired on proteins is a relevant strategy since 3,020 proteins were identified, representing the largest protein inventory for this sentinel organism. Functional annotation and gene ontology (GO) analysis performed on the identified proteins described the main molecular players of hemocytes and plasma in immunity. GO analysis highlights the complementary immune functions of these two compartments in the management of micro-organisms. Functional annotation revealed new mechanisms in the immune defence of the zebra mussel. Proteins rarely observed in the hemolymph of bivalves were pinpointed such as natterin-like and thaumatin-like proteins. Furthermore, the high abundance of complement-related proteins observed in plasma suggested a strong implication of the complement system in the immune defence of *D. polymorpha*. This work brings a better understanding of the molecular mechanisms involved in zebra mussel immunity.

Significance

Although the molecular mechanisms of marine bivalves are widely investigated, little information is known for continental bivalves. Moreover, few proteomic studies described the complementarity of both hemolymphatic compartments (cellular and plasmatic) in the immune defence of invertebrates. The recent proteogenomics concept made it possible to discover proteins in non-model organisms. Here, we propose a proteogenomic strategy with the zebra mussel, a key sentinel species for biomonitoring of freshwater, to identify and describe the molecular actors involved in the immune system in both hemocytes and plasma compartments. More widely, this study provided new insight into bivalve immunity.

1. Introduction

Immune system acts as the main defence mechanism against invasive organisms. In bivalves, the immune defences are mainly undertaken in the hemolymph compartment. This circulatory fluid comprises immune cells, the hemocytes, and a cell-free compartment, the plasma. Innate immune system process of bivalves can be summarized in three main steps: (i) the recognition of molecular motifs associated with microorganisms (MAMPs) or endogenous molecules secreted by damaged tissues (DAMPs) by soluble compounds and cellular receptors, (ii) the activation of different signalling pathways, (iii) the production of molecular effectors involved in host defense and cellular defence responses [1].

Due to their economic interest and the disease outbreaks faced in aquaculture, molecular immune system of marine bivalves are well documented compared to continental bivalves which are mainly used to assess water quality. Among continental bivalves, the freshwater mussel, *Dreissena polymorpha*, represent the counterpart of the marine mussel in ecotoxicological studies [2]. While cellular immune responses of hemocytes are well characterized for *D. polymorpha*, few information are available on molecular immune responses [3]. As a non-model species, no complete genome is currently available for *D. polymorpha* and genetic markers are relatively scarce, especially for immune related genes. To our knowledge, only a single study reported about thirty genes involved in hemocyte responses against microbial antigens [4]. These genes, such as ATP synthase or Heat Shock Protein 70, were more related to general stress responses than immune system defence.

To date, research on bivalve immunology mainly focused on immune responses of hemocyte using transcriptomic investigations [5]. Because plasmatic compartment is essentially mRNA-free, such approach does not give a comprehensive picture of the entire hemolymph compartment. Regarding the proteome of the hemolymph of aquatic invertebrates, the information remains scarce because only 2D gel electrophoresis results have been till now reported [5]. This approach focuses only on the most abundant and soluble proteins [6], explaining the relatively low number and low relevant proteins identified in bivalve plasma.

Next-generation sequencing technologies have made great progress over the last decade, providing information on i) structure and molecular function of the genetic material, and ii) level of expression of genes. Proteins and metabolites are important components of biological systems that can also be assessed at large scale by state-of-the-art tandem mass spectrometry. The recent proteogenomics concept emerged as a relevant strategy for the discovery of proteins in non-model organisms [7]. For animals and plants, this approach combines data obtained by high-throughput mRNA sequencing (RNA-seq) for establishing the most appropriate protein sequence database and large-scale protein shotgun sequencing by tandem mass spectrometry. Indeed, peptide sequence identification relies on the comparison of experimental peptide mass spectra obtained by tandem mass spectrometry against all the possible theoretical spectra derived from the protein sequence database. For non-model organisms whose genome have not yet been sequenced, it has been proposed the construction of a theoretical protein sequence database on the basis of the sequenced transcriptome *in silico* translated to amino acid sequences in all three possible reading frames. Proteogenomics has already proven its capacity in quickly documenting proteins of non-model sentinel species used for continental water biomonitoring. For example, 1,873 proteins related to the reproductive system of *Gammarus fossarum* were experimentally validated using proteogenomics [8].

In order to get insights into the immune proteome of the zebra mussel, proteogenomics was conducted on both hemocytes and plasma compartment. For this, RNAseq and shotgun mass spectrometry with a tandem mass spectrometer equipped with an ultra-high-field Orbitrap analyzer were performed on the *D. polymorpha* sentinel species. Both data were combined leading to the identification of 3,227 novel proteins. Proteins involved in immune processes and potential immune proteins of both compartments were highlighted and commented. This study contributes to a better understanding of the molecular mechanisms involved in immunity of bivalves.

2. Material and Methods

2.1 Construction of a paired-end RNA-Seq library and illumina sequencing.

Zebra mussels, *Dreissena polymorpha*, between 15- and 25-mm long were collected at the lake of Der (Grand Est, France) in July and September 2016. Dissection of i) gonad male and ii) female, iii) gills, iv) digestive gland, v) whole tissues and sampling of vi) total hemolymph was carried out directly after harvest. To avoid natural individual variation, six organs of each type were pooled, frozen immediately in liquid nitrogen and stored at -80 °C until total RNA extraction. Pools of hemolymph were centrifuged twice at 3,000 x g for 5 min at 4 °C to separate the hemocytes from the plasma. Pellets of hemocytes were mixed with 500 µL of Trizol reagent (Sigma-Aldrich, Saint-Quentin Fallavier, France) in order to disrupt

cell walls. Tissue samples were disrupted with 1 ml of Trizol reagent (Sigma-Aldrich) per 100 mg of tissue and subjected to an ultra-turrax shredder run at 20,000 rpm for 5 min.

Total RNA of each sample was isolated according to Sigma-Aldrich instructions. Briefly, samples were incubated at room temperature for 5 min. Then, a volume of 200 μ l of chloroform was added to homogenize by shaking during 30 sec. After 15 min at room temperature, samples were centrifuged at 12,000 x *g* for 15 min at 4 °C. The aqueous phase was introduced into a new tube and 500 μ l of isopropanol was added. After 10 min of incubation, samples were centrifuged at 12,000 x *g* for 8 min at 4 °C. RNAs were washed with 1ml of 70% ethanol, followed by centrifugation at 7,500 x *g* for 5 min at 4 °C. RNAs were dried, and then samples were resuspended in 40 μ l of RNase-free water and incubated in a water bath at 57 °C for 15 min. Total RNAs for each sample were quantified using a Genova Nano spectrophotometer (Jenway, UK) and their qualities were checked using agarose gel. Samples were stored at -80°C until RNAseq library construction.

RNA Seq library construction and sequencing were performed by Acobiom (Montpellier, France), using the HiSeq2000 sequencing instrument (Illumina). A total of six RNAseq libraries, corresponding to each tissue sampled, were constructed according to the TrueSeq Stranded (Illumina) protocol with 500 ng of total RNAs. Samples were sequenced using paired-end reads 2x150 bp method and the reads were assembled with the software Trinity pipeline (2.3.0 version) following the procedure of Haas et al. [9]. A transcriptome reference, called "Dreissena_reference", was constructed by compiling the 6 RNAseq data.

2.2 Sample preparation for mass spectrometry analysis

Zebra mussels between 15- and 25-mm long were collected at the lake of Der (Grand Est, France) in May 2017. Mussels were maintained for at least two weeks at the laboratory in 20 L tanks filled with spring water Cristaline Aurèle (Jandun, France), with a constant temperature (14°C) and controlled aeration. Mussels were fed twice a week with a mixture of two species of microalgae, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (two millions of microalgae per day and per mussel). Tank water was removed weekly and replaced by fresh water. After the acclimation period, 200 μ L of hemolymph per mussel was collected from the posterior adductor muscle by gentle aspiration with a 30 G x 0.3 mL syringe. After checking hemolymph quality under a binocular microscope, hemolymph samples were pooled to reach 1 mL hemolymph per replicate, which represents a minimum of five and a maximum of eight mussels per pool of hemolymph. Five biological replicates were performed. Pools of hemolymph were then centrifuged at 800 x *g* for 15 min at 4 °C to gently separate the hemocytes from the plasma. Hemocyte pellets were mixed with 500 μ L of Tri-reagent (Sigma-Aldrich) and stored at -20°C until the protein extraction step while the resulting supernatant (plasma) was immediately added to 3 volumes of ice-cold acetone and stored overnight at -20 °C to allow protein precipitation. Precipitated proteins of plasma were centrifuged at 15,000 x *g* for 10 min at 4 °C. The protein pellets were washed twice

with 1 mL of 80 % cold acetone followed by centrifugation at 15,000 x *g* for 10 min at 4 °C, and removal of the supernatant. Then, the pellets were dried at room temperature and re-suspended in 80 µL of re-suspension buffer consisting of 6 M urea; 2 M thiourea ; 4 % 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS); 65 mM dithiothreitol (DTT), 0.1 % Triton X100 (Vol:Vol), pH 7.8.

Hemocytes proteins were extracted following the procedure described by Vaibhav et al. [10] with few modifications. Briefly, hemocytes were lysed by passing them ten times through a syringe equipped with a 25 G needle and centrifuged at 12,000 x *g* for 2 min for an initial clean up. RNAs were removed from the resulting supernatant by adding 100 µL of chloroform, incubating for 15 min, centrifugation at 12,000 x *g* and 4 °C for 15 min, and removing the colorless aqueous phase. DNA was discarded by adding 150 µL of absolute ethanol for 3 min followed by centrifugation at 2,000 x *g* and 4 °C for 5 min. Then, proteins from the resulting supernatant were precipitated by adding 3 volumes of ice-cold acetone to the supernatant. Samples were incubated at room temperature for 10 min and then centrifuged for 10 min at 12,000 x *g* and 4 °C. The remaining protein pellet was then washed three times for 10 min in 1 mL of 0.3 M guanidine hydrochloride in 95 % ethanol (Vol:Vol) followed by centrifugation at 8,000 x *g* and 4 °C for 5 min and removal of the supernatant. A final wash was performed with 1 mL of 95 % ethanol before protein pellets were dried at room temperature and re-suspended in 80 µL of resuspension buffer.

The protein concentration of each sample was measured by Bradford assay with a calibration curve established with bovine serum albumin (BSA). Biological replicates were diluted in re-suspension buffer to obtain the same protein concentration of 2.25 µg/µL. A volume of 20 µL of each sample was diluted with 10 µL of LDS 3X buffer (Invitrogen) consisting of 80 mM Tris / HCl, 106 mM Tris base, 1.5 % lithium dodecyl sulfate, 7.5 % glycerol, 0.38 mM EDTA, 0.17 mM SERVA Blue G250, 0.131 mM phenol red, buffered at pH 8.5 and supplemented with 2.5 % beta-mercaptoethanol. Then, samples were boiled for 5 min at 95 °C, centrifuged briefly, and loaded onto a 4–12 % gradient 10-well NuPAGE gel (Invitrogen). A 5 min electrophoretic migration of the proteins was performed at 200 V in MES buffer (Invitrogen). After Coomassie Blue Safe staining (Invitrogen) and destaining with ultra-pure water, each polyacrylamide band corresponding to each whole proteome was excised. Proteins from each polyacrylamide band was first alkylated with iodoacetamide, reduced with DTT and then proteolyzed with Sequencing Grade Trypsin (Roche) in presence of 0.01 % ProteaseMAX surfactant (Promega) for 1 h at 50 °C as previously described by Hartmann et al. [11]. The proteolysis was then stopped with the addition of TFA 5 % to reach a final concentration of 0.5 %.

2.3 NanoLC-MS/MS analysis

The resulting peptide mixtures were analyzed in a data-dependent mode with a Q Exactive HF tandem mass spectrometer (ThermoFischer Scientific) coupled on line to an Ultimate 3000 chromatography system (ThermoFisher), essentially as previously described [12]. A volume of 5 μ L of each sample was injected, first desalted with a reverse-phase capillary precolumn C₁₈ PepMap TM 100 (LC-Packing) and then resolved with a nanoscale 500 mm C18 PepMap TM 100 (LC-Packing) column using a 90 min gradient from 20 % to 80 % of CH₃CN, 0.1 % formic acid. Mass spectra of peptide ions with 2+ and 3+ charge states were acquired in the full scan range m/z 350-1800, with a resolution set at 60,000, an AGC target at 3×10^6 , following a Top20 method for selecting peptides for fragmentation. MS/MS mass spectra were acquired with an AGC target set at 10^5 , a loop count of 60 msec, an isolation window of 1.6 m/z , a resolution at 15,000, and a scan carried out in a mass range from 200 to 2,000 m/z .

2.4 Proteogenomics database design and MS/MS interpretation

The Dreissena_reference transcriptomic database was translated into the six possible reading frames with a homemade python script. The resulting translated contig database contained 5,648,820 polypeptide sequences, totaling 941,472,234 residues. MS/MS spectra were assigned to peptide sequences with the Mascot search engine with the following parameters: full-trypsin specificity, a maximum of two missed cleavages, a mass tolerance of 5 ppm on the parent ion and 0.02 Da on the MS/MS analysis, and a pvalue for peptide validation at 0.1. Modifications taken into account were carboxyamidomethylated cysteine (+57.0215) as static modification and oxidized methionine (+15.9949) and deamidation of asparagine and glutamine (+0.9848) as dynamic modifications. Translated contigs identified with at least one MS/MS certified peptide were selected for creating a reduced database named "Dreissene_cascade". This second database comprised 54097 polypeptide sequences, totaling 20,419,358 residues. Then, a second Mascot round of search was performed with a p value threshold for validating peptide assignment set at 0.05. At this stage, a protein was validated when at least two unique peptide sequences were detected in at least one of the ten samples used in this study. The false positive rate for protein identification was estimated, through a search with a reverse decoy database, as less than 1% using the same parameters. For each protein and for each replicate, the numbers of spectral counts observed for the different detected peptides and corresponding to a peptide-to-spectrum assignation were summed. Then, the spectral counts detected in each of the five biological replicates were merged to obtain the spectral counts of each protein in each of the two conditions, hemocytes and plasma. As previously proposed [13], the normalized spectral abundance factor (NSAF) of each protein was calculated as the sum of the spectral counts (SC) observed in the five replicates of hemocytes or plasma, divided by its molecular mass expressed in kDa. The NSAF values were then presented as a ratio (%NSAF) by dividing the NSAF of each protein to the sum of all NSAF values detected in

hemocytes or plasma. Spectral count missing values are observed in the case of low abundant proteins, no specific correction of missing values was made.

2.5 Protein functional annotation and gene ontology analysis

Functional annotation of MS/MS identified proteins was derived from a sequence similarity search without taxonomical restriction against (i) the National Center for Biotechnology Information (NCBI) database of non-redundant protein sequences (nr) (131,025,392 sequences released 05-09-2017) and (ii) the Swissprot database (465,089 sequences released, 30-09-2016), using the blastp module of DIAMOND (version 0.8.22) with an E-value threshold set at 1E-03. Functional annotations were further confirmed by searching conserved domain (CD) of proteins with the Batch CD-Search tool of NCBI server [14]. Gene Ontology (GO) assignment for each translated contig was obtained with the GO database (<http://www.geneontology.org/>). GO assignments were used to classify molecular processes and biological processes of the hemocyte and plasma proteins. A focus on immune-related proteins was performed by studying GO annotation of the proteins that matched with the GO-term “immune system process”.

2.6 Mass spectrometry and proteomics data

The mass spectrometry and proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [15] partner repository with the dataset identifier PXD012991 and 10.6019/PXD012991.

3. Results and discussion

3.1 A double round search for proteogenomics identification of proteins from the hemolymph of *D. polymorpha*.

We applied a proteogenomics-derived strategy for quickly identifying the proteins from two fractions of the hemolymph of *Dresseina polymorpha* physiologically acclimated to laboratory conditions. Figure 1 shows a schematic representation of the experimental strategy and the double round searches for interpreting the tandem mass spectrometry data. First, RNAseq was performed on six samples of mRNAs extracted from six different tissues of zebra mussels: i) total hemolymph, ii) gonad male and iii) female, iv) gills, v) digestive gland and vi) whole tissues, as indicated in Figure 1. A total of 160,869,134 paired-end reads of 150 bp were assembled into transcripts with the Trinity software resulting in a list of 941,470 contigs with a N50 of 594. A global proteogenomics reference database “Dreissena_reference” was created consisting of the systematic translation of all these

contigs into the six possible frames. A dataset that will be described elsewhere (Lepretre et al., unpublished data) and comprising 3,211,276 MS/MS spectra recorded on the proteomes of various hemocytes and plasma samples was interpreted against the “dreissena_reference” RNAseq-derived database to select relevant contigs for creating a reduced database as previously recommended [16]. The attribution rate was 30% for hemocytes and 29% for plasma, a result similar to other proteogenomics analysis performed on non-model organisms with inflated RNA-seq proteogenomics database [17]. The resulting reduced database comprised 54,097 protein sequences, a size comparable to classical animal proteomics based on a sequenced and annotated genome. For the present study, five biological samples of hemolymph were treated to get five fractions of hemocytes and five fractions of plasma. Their protein content was explored by next-generation shotgun proteomics resulting in a total of 281,799 MS/MS spectra recorded for the hemocytes and 261,188 MS/MS spectra for the plasma. As indicated in Figure 1, the second round of search resulted in the interpretation of 103,979 MS/MS spectra (37%) and produced a list of 22,578 unique peptide sequences leading to the discovery of a total of 3,020 proteins in the hemolymph (hemocytes and plasma), validated by at least two unique peptide sequences.

A higher number of proteins were identified in hemocytes compared to plasma (Fig. 2A). 1,489 proteins were assigned exclusively to the hemocytes and 840 proteins were observed only in the plasma compartment while 691 proteins were identified in both compartments. Separation process of hemocytes from plasma by centrifugation may produce cell lyses leading to a protein contamination of plasma. In this case, some of the proteins identified with a higher NSAF% in hemocytes compared to plasma could be exclusive to hemocytes. 433 proteins were following this trend, which represent 60% proteins of the 691 proteins observed in both hemocytes and plasma. Because the same efforts in terms of tandem mass spectrometry have been done for the two kinds of samples, results are probably directly correlated to the dynamic range of these proteomes. A similar trend was observed in the proteome of hemolymph from the marine mussel *Mytilus edulis* [18]. The authors identified a total of 654 proteins in the hemolymph, 321 proteins were observed exclusively in hemocytes, 178 were exclusively assigned to the plasma and 178 were common to both compartments. Functional annotation of these proteins was performed by sequence similarity search against the NCBI nr and Swissprot databases. Most of the proteins (2,948) could be functionally annotated, but a relatively small set of protein sequences (2 %) did not present any significant sequence similarities with known proteins and thus could be considered as orphan proteins. Regarding the species origin of the most similar BLAST hit against NCBI nr, the majority of proteins showed as expected strong sequence similarities with proteins from molluscs taxa (Fig. 3). The pacific oyster *Crassostrea gigas* is the most phylogenetic closely-related species based on this comparison with a total of 750 proteins with high similarities to *D. polymorpha* MS/MS-detected proteins, followed by another marine bivalve *Mizuhopecten yessoensis*. Due to their economic importance in aquaculture, these two bivalves are highly studied and a large number of genomic resources are available, making them the closest genetic resources to the zebra mussel known to date [19]. As

expected, *D. polymorpha* was poorly represented with only 8 proteins as its genome is not yet sequenced and only a restricted set of proteins are till now present in the NCBI nr database.

3.2 Gene ontology analysis of the immune proteome

A Gene ontology analysis was performed on the whole set of mass spectrometry certified proteins in order to describe biological processes and molecular functions of the zebra mussel's proteins from the hemolymph compartment. Focusing on the immune system, 563 proteins in hemocytes against 490 in plasma were assigned to the GO-term "Immune system process" (Fig. 2B). Among them, 216 immune-related proteins were observed in both hemocytes and plasma compartments, representing almost half of the immune proteins identified in plasma. To understand the role of hemocyte and plasma compartments in *D. polymorpha* in the management of immune responses, a gene ontology (GO) analysis was performed on the set of proteins mapped with the GO-term "immune system process", which were considered as immune proteins (Fig. 4). For this proteomics analysis, we choose a label-free comparison of the two compartments based on spectral count as this method is relatively independent of the ionizability variability of peptides. Furthermore, we systematically normalized the spectral counts taking into account the mass of each polypeptides, thus allowing a comparison of the abundances of various proteins. In this context, Go-term annotations were weighted with NSAF values in order to compare both compartments in terms of abundances.

Results of biological process Go-terms showed a higher NSAF value of immune proteins of plasma (0.72) compared to hemocytes (0.45). While overall the same trend of biological process and molecular function was observed between the hemocyte and plasma compartments, few differences could be noticed (Fig. 4). The most represented biological process of immune proteins was "response to stress", with a NSAF twice as important in plasma (0.66) compared to hemocytes (0.32). Such results confirm that immune responses occurring in plasma and hemocytes are highly sensitive to stressful conditions, making them a relevant marker for ecotoxicological studies. Another highly represented biological process was "vesicle-mediated transport" in both compartments. Exocytosis of immune proteins from the hemocytes to extracellular region plays a critical role in the elimination of foreign particles. This process occurs principally through vesicle-mediated transport, which mediates the release of proteins in the extracellular space that participate in immune signalling, recognition and destruction of micro-organisms [20]. The biological process "signal transduction" was also prominent in both hemocyte and plasma compartments. Signal transduction is required for the modulation of immune responses. This process is initiated by the recognition of MAMPs or DAMPs by cellular receptors and/or soluble compounds localized in cells and extracellular compartments. Immune signalling process plays a central

role by controlling effective immune responses against microorganisms. Immune signalling pathways have been largely documented in hemocytes of bivalve species [21]. In plasma, immune signal transduction also plays important role in controlling the defence responses of bivalves. For example, in molluscs, the extracellular signal-regulated kinase (ERK) signalling pathway regulates many immune process of hemocytes, including inflammation, proliferation and apoptosis [22]. In both compartments, the GO analysis revealed a high NSAF value of immune proteins related to membrane organization, cell motility and cell adhesion. Recruitment and migration of hemocytes to the site of infection is one of the key steps of the immune response. These processes involve a dynamic cytoskeleton remodeling and shape changes of hemocytes, mediating cell motility, cell adhesion and endocytosis/phagocytosis of foreign particles [23]. Nitrogen compound metabolic process and cell death process were also highly represented in both compartments. This is not surprising since molluscs produce nitrogen species for their basal metabolism as well as in response to exogenous stimuli and programmed cell death is involved in homeostasis of the immune system [24]. In hemocytes, a higher NSAF of immune proteins was observed for the GO-terms related to cytoskeleton organization, cell cycle and autophagy, this latter is considered as an important immune process of bivalve's hemocytes [25]. Interestingly, some immune proteins were related to neurological system process in both compartments. While the nervous system of invertebrates is poorly studied, Malagoli and Ottaviani [26] evidenced a strong complementarity between the nervous system and immune system of mollusc species.

Regarding molecular functions of immune proteins, more differences were observed between the two compartments (Fig. 4B). Molecular function related to peptidase activity was the most represented in the hemolymph with a NSAF value four times higher in plasma compared to hemocytes. Similarly, immune proteins related to enzyme regulator activity were more represented in plasma than hemocytes (0.34 in plasma and 0.08 in hemocytes). Proteolytic enzymes are considered as integrative components of the bivalve defence system [1]. They are involved in various immune system processes, such as immune signalling, cell death and degradation of microbial cell wall components [27,28]. The plasma compartment of bivalves is an important source of proteolytic enzymes that govern immune responses. For example, proteolytic cascades are essential to the activation of immune responses occurring in the plasma of bivalves, such as the complement system and the prophenoloxidase systems [1]. Finally, immune proteins with functions associated with general cell metabolism were more represented in hemocytes compared to plasma. In this group are included proteins assigned to ATPase activity, phosphatase activity, and ligase activity (Fig. 4B).

3.3 Identification of immune-related proteins of the hemolymph

According to the literature, several proteins were identified as potentially involved in the immune defence of zebra mussel. In this section, a focus will be given on the proteins involved in the (i) recognition of microorganisms, (ii) immune signalling, (iii) micro-organisms internalization, (iv) elimination/destruction of microorganisms, and (v) apoptosis. It is important to highlight that the proteins described in this chapter are proteins known to be involved in the immune defence of organisms different than zebra mussels. Further investigations with mussels challenged with immuno-modulators are necessary to validate their involvement in *D. polymorpha* immunity. Whereas most of the immune proteins were shared between hemocyte and plasma compartments some of them appeared to be specific of one compartment. Informations on the functional annotation and abundance of identified proteins in hemocytes and plasma are detailed in the supplement Table S1 and Table S2 respectively. Supplement Table S3 details conserved domain (CD) of proteins, which supports the functional annotation.

3.3.1 Recognition of micro-organisms

Several proteins acting as pattern recognition receptors (PRRs) were found in high abundance in the hemolymph of *D. polymorpha*. In bivalves' immunity, PRRs are often associated with host defence responses against infection [1]. They allow the recognition of MAMPs or DAMPs, which lead to the activation of different immune processes. Interestingly, a natterin-like protein containing a jacalin-lectin domain and a pore-forming toxin domain was identified as a major protein in the zebra mussel plasma (898 SCs) and hemocytes (804 SCs), which represents about 2.5% of the proteome in both compartments (Table 1). To our knowledge, this is the first time that a natterin-like protein is identified in the hemolymph of invertebrates. Natterins were first described as the major toxins from the venomous fish *Thalassophryne nattereri*. According to the literature, this proteolytic enzyme could play an important role in the innate immune process. Firstly, a kinogenase activity was characterized in natterins purified from *T. nattereri* [29]. Further investigations showed that natterins exert cytotoxic effects and can induce cell necrosis by degrading or remodelling extracellular matrix [30]. Looking at its structure, a pore-forming aerolysin domain and a N-terminal domain were identified in natterin genes isolated from the blood of lamprey (*Lampetra japonica*) and the skin mucus of the Atlantic cod (*Gadus morhua*) [31,32]. Taken together, these findings suggest that natterins act as pathogen recognition proteins with potential toxin function.

In total, 4,962 SC were attributed to proteins belonging to the lectin family in the *D. polymorpha* hemolymph (Table 1). Made of carbohydrate recognition domains (CRD), lectins enable the recognition of the "non-self" carbohydrate patterns localized on the surface of microorganisms such as glycans of parasite, bacteria lipopolysaccharide or glycoproteins of the viral envelope, leading to the internalization and destruction of invaders by phagocytic cells [33]. Lectins have been classified into several families depending on their structural fold

of carbohydrate recognition domain. In *D. Polymorpha*, C-type lectins (CTLs), R-type lectins, galectins and fibrinogen-related proteins (FREPs) were found in both hemocytes and plasma while a P-type lectin was exclusively found in plasma. CTLs were five times more abundant in plasma (956 SCs) in comparison to hemocytes (169 SCs) (Table 1). The function of CTLs in the immune system of molluscs has been firmly established. Several studies have demonstrated the involvement of CTLs in the recognition, immobilization and opsonization of microorganisms in the plasma and hemocytes of many mollusc species [34–36]. Furthermore, the expression of CTLs in hemocytes was modulated during an infectious experiment, which confirms their involvement in the defence against pathogens [37]. Mytilecs proteins are R-type lectins isolated from the Mediterranean mussel, *Mytilus galloprovincialis*. The structure of Mytilecs is composed of a glycan-binding domain and a pore-forming aerolysin-like domain [38]. Like natterins, in addition to its pathogen recognition function, the aerolysin-like domain of Mytilec are potentially directly devoted to kill micro-organisms [39]. Two proteins of the zebra mussel hemolymph showed sequences similarities with Mytilecs proteins. One was observed in high abundance in both hemocytes and plasma while the other Mytilec, which contains an aerolysin-like domain, was only found in plasma. Galectins were also observed in high abundance in the intra- and extra-cellular compartments of zebra mussel hemolymph (588 SCs in hemocytes and 484 SCs in plasma) (Table 1). Invertebrate galectins have been shown to play crucial roles in innate immunity. In addition to their role in the recognition of MAMPs, galectins participate to many immune processes such as cell adhesion, phagocytosis, immune regulation and pathogen destruction [40–42]. 222 and 428 spectra associated with proteins containing fibrinogen-related domains (FreDs) were identified in hemocytes and plasma respectively. While these lectin-like molecules are known to have coagulation functions in vertebrates, the primary role of invertebrates FreDs is immune recognition of microorganisms [43]. For example, FreDs in the hemolymph of the bay scallop *Argopecten irradians* were significantly up-regulated after a gram-negative bacteria infection and presented agglutinating properties, emphasizing the contribution of FREPs to non-self recognition in invertebrates [44]. Other lectins type such as mannose-6-phosphate-receptor were identified exclusively in plasma (57 SCs) (Table 1). Cation-dependent mannose-6-phosphate receptor (MPR) are P-type lectin acting as PRR. Zhang et al. [45] showed that MPR of shrimp was able to recognize invading bacteria and stimulate the production of antimicrobial peptides in the hemolymph of the shrimp *Marsupenaeus japonicus* during *in vivo* challenges with gram-positive and gram-negative bacteria.

Other immune PRRs, were found in the hemolymph of *D. polymorpha*. A Beta-1,3-glucan-binding protein (GBP) was identified in both compartments of the hemolymph of *D. polymorpha* with more than 200 spectra counted in both compartments and a peptidoglycan-recognition protein (PGRP) was found ten times more abundant in plasma compared to hemocytes (Table 1). GBP act as a PRR by recognizing β -1, 3-glucan molecules of the bacteria cell wall and trigger the innate immune responses [46]. PGRP are secreted proteins which have been characterized in the hemolymph of oyster and scallops, and their

expression were found enhanced after bacteria challenges [47,48]. This PRR has a lysozyme domain, which may confer bacteriolytic activity in addition to the peptidoglycan binding capacity [47].

3.3.2 Proteins involved in immune signaling

Immune signalling is a crucial step in adapting an effective immune response leading to the destruction of different kinds of microorganisms. Few proteins involved in immune signalling of *D. polymorpha* were observed in plasma compared to hemocytes (Table 1). Of them, two proinflammatory cytokines named allograft inflammatory factor 1 (AIF-1) and macrophage migration inhibitory factor (MIF) were identified with a higher abundance in hemocytes (84 SCs) compared to plasma (43 SCs) (Table 1). AIF-1 and MIF have been identified as regulator molecules of the immune system of bivalve species. MIFs were modulated upon bacterial challenge in the hemocytes of the clam *Ruditapes philippinarum* and the scallop *Chlamys farreri* [49,50]. Both studies concluded that MIF cytokines were involved in proinflammatory responses by promoting hemocyte migration to the wound healing site. AIF-1 was also up-regulated under several microorganism challenges and tissue injury in the hemocytes of bivalves [51–53]. Like in vertebrates, AIF-1 of bivalves promoted inflammatory processes and stimulated phagocytosis of foreign microorganisms.

Mitogen-Activated Protein Kinases (MAPKs) are important proteins involved in the regulation of cellular processes including cell proliferation, apoptosis and immune response, especially against virus, fungi and bacteria [54]. Till now, three major MAPKs pathways have been identified in the hemolymph of bivalves, the c-jun N-terminal kinases (JNKs), p38 mitogen-activated protein and the extracellular signal-regulated kinases (ERKs) [33]. Several studies showed the implication of these MAPKs pathways in the regulation of immune signal transduction, leading to the production of immune effectors adapted to the infectious pathogen [22,55,56]. In the hemolymph of *D. polymorpha*, MAPKs were evidenced by 320 spectra in hemocytes and 80 spectra in plasma (Table 1). Key components of ERKs (ERK1, ERK2) and p38 mitogen-activated protein (p38-MAPK and MAP2K-6) were identified in hemocytes and plasma, while a component of the JNK pathway (stress-activated protein kinase JNK) was exclusively identified in hemocytes.

The interferon signalling pathway is considered as a powerful antiviral defence of the innate immune system [57]. While the main anti-viral defence in bivalves was associated with the blocking of viral replication by RNA interference, recent studies evidenced interferon (IFN) signalling pathway similar to vertebrates to counteract virus invasion [58–60]. Briefly, the recognition of double-strand virus by PRRs leads to the activation and expression of interferons, resulting to the activation of signal transducers and activators of transcription (STATs), which stimulate the production of antiviral effectors such as the interferon-induced protein 44-like (IFN-44) [58,61]. In our study, IFN-44 and a STAT-related

protein were exclusively identified in the hemocytes of the zebra mussel with 97 SCs. Their detection suggests the involvement of IFN pathway in antiviral defences of *D. polymorpha* (Table 1).

Other proteins involved in immune signalling, such as the nuclear factor kappa B (NF- κ B), protein kinase C -type (PKC) and phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K), were identified in the hemolymph of *D. polymorpha*. The transcription factor NF- κ B was already reported to be present in hemocytes of many bivalve species and was considered as a key regulator of the immune response by its participation in the production of immune effectors, immune cytokines and proinflammatory factors [33]. The protein kinase PKC and PI3K are important regulators of phagocytosis process of bivalve hemocytes [62].

3.3.3 Proteins involved in the internalization of microorganisms

Internalization of microorganisms by hemocytes is a fundamental process of innate immune defence, causing the destruction of invaders. Internalization process consists of three main stages: (i) the cell migration towards the microorganisms, (ii) cell adhesion and (iii) internalization. While these three main stages are well characterized at the cellular level in many invertebrates, little is known on the molecular actors that govern these responses. To date, most of the proteins known to be involved in internalization processes are related to the cytoskeletal regulatory proteins [63]. Indeed, cell migration, cell adhesion and internalization require actin polymerization, which generates mechanical forces and morphological changes to meet and ingest invaders [64].

Several studies have highlighted the central role of the actin-related protein 2/3 complex (Arp2/3) in actin filament assembly during migration and phagocytosis process of macrophages [64–66]. The activation of the Arp2/3 complex is carried out locally under the action of proteins of the WASP family (Wiskott-Aldrich syndrome protein). These latter are activated at the cell membrane by small GTPases of the Rho family such as Cell Division Cycle 42 (CDC42) and Ras-related C3 botulinum toxin substrate (Rac1). In invertebrates, the implication of Arp2/3 complex in cell motility and phagocytosis by hemocytes have been evidenced in *Drosophila* and oyster [65,69]. For example, Lau et al. [67] observed an up-regulation of proteins involved in the regulation of arp2/3 complex associated with an increase in hemocyte motility when oysters were challenged with the parasite *Perkinsus marinus* and latex beads. In the hemolymph of *D. polymorpha*, many proteins involved in the regulation of the Arp2/3 complex were identified. CDC42, RAC1, and Arp2/3 were detected in both compartments with higher abundance in hemocytes compared to plasma while neural Wiskott-Aldrich syndrome protein (NWASP) was exclusively observed in the hemocytes.

Integrin subunits alpha and beta were eleven times more represented in hemocytes (941 SCs) compared to plasma (85 SCs) (Table 1). Probably, their presence in plasma may be the

result of plasma contamination by hemocytes during centrifugation process. These transmembrane adhesion receptors play critical roles in the innate immune process such as cell migration, cell attachment with pathogens, phagocytosis and apoptosis of immune cells (reviewed in [68]). The implication of integrins in the internalization of microorganisms has been documented in several invertebrate species, including bivalves [69–71]. For example, integrins were up-regulated during bacterial infection in the Pacific oyster hemocytes [72]. The authors showed that the phagocytosis rate of bacteria decreased significantly after integrin blocking with antibodies, which evidences the involvement of integrins in the internalization of pathogens.

Proteins with functions related to clathrin-mediated endocytosis, including clathrin heavy chain and adaptor proteins (APs), were produced mainly in the zebra mussel hemocytes (349 SCs) (Table 1). Cellular uptake by the clathrin-mediated endocytosis is crucial for many cellular functions such as nutriment uptake, cell homeostasis and growth control [73]. Moreover, the clathrin-mediated pathway is used by viruses to enter into the cells [74,75]. Binding of viral particles to cell receptors triggers the accumulation of key components of clathrin-mediated endocytosis including clathrin, AP2 and dynamin, which leads to clathrin coat assembly and cargo internalization [75]. The clathrin-mediated endocytosis of viruses has also been demonstrated in the hemocytes of *Marsupenaeus japonicus* and *Penaeus monodon* shrimps [76,77].

Other proteins with functions related to cytoskeleton remodeling and cell motility were detected in the zebra mussel hemocytes. In this group we included dedicator of cytokinesis protein 1, myosin II and Rho-associated protein kinase 2.

3.3.4 Humoral Immune Effectors

Humoral Immune effectors, antimicrobial peptides, proteases and protease inhibitors are viewed as key elements of the innate immunity of bivalves since they participate to the degradation of specific substrates including microbial cell walls [1].

As the first line of defence of the innate immune system of shellfish, antimicrobial peptides (AMPs) are immediate, rapid and powerful weapons to protect organisms against invasive pathogens. A thaumatin-like protein (TLP), containing a thaumatin domain, was the most abundant protein considered as AMP in both hemocytes (201 SCs) and plasma (149 SCs) of *D. polymorpha* (Table 1). This antifungal protein has been discovered in a wide range of organisms, including plants, fungi and insects [78]. TLPs are believed to exert antifungal activity using a variety of enzymatic activities, which make them a potent AMP. Indeed, TLPs have been shown to exert glucan binding and β -1,3-glucanase activities in addition to their function as glucose polymers degrading, chitinase, xylanase, as well as α -amylase inhibiting activities [78]. For bivalves, information concerning TLPs is still very limited. To our knowledge, TLPs were only identified in hemocytes of the freshwater Pearl mussel (*Hyriopsis*

cumingii) and in mucosal secretions in the eastern oyster, *Crassostrea virginica* [79,80]. A Defensin-like protein was identified exclusively in the hemocytes of the zebra mussel (Table 1). Defensins are a family of antimicrobial peptides found in many organisms, including plants, vertebrates and invertebrates [81]. These are small cationic peptides which are widely involved in innate immunity due to their antimicrobial activity [81]. Defensins have been identified in several bivalve species and were shown to be active against a wide range of bacteria, protozoan and fungi [82,83]. A defensin-like protein had already been identified in tissues of the zebra mussel, including hemocytes [84]. Authors showed that the expression of the *D. polymorpha* defensin from hemocytes exerted antimicrobial activity against gram-negative bacteria during *in vitro* challenges. Other proteins considered as part of the antimicrobial arsenal such as lysozymes, bactericidal/permeability-increasing protein (BPI) and histones were also observed in the hemolymph of *D. polymorpha*. Lysozymes were mainly identified in plasma with 51 SCs (Table 1). Two type of lysozymes were observed, a C-type lysozyme (Chicken type) lysozyme and an I-type lysozyme (Invertebrates-type) containing a destabilase domain. Lysozymes are part of the family of glucoside hydrolases, they are found in a wide variety of tissues and secretion from vertebrates to invertebrates [85]. The bacteriolytic enzymes I-type lysozymes are well documented in invertebrates and have been found to play a major role in the destruction of bacteria due to their antimicrobial activities [85]. In contrast, the functions of C-type lysozymes have been poorly studied in bivalves [85]. To date, C-type lysozymes were only identified in tissues of the mussel and displayed lytic activities against Gram-positive and Gram-negative bacteria [86]. BPI protein was exclusively observed in the hemocytes of the zebra mussel with a low abundance. BPIs are strong pore-forming agents exerting antibiotic action towards Gram-negative bacteria. A BPI has been characterized in the hemocytes of *C. gigas* oyster [87]. Its pattern of expression and its ability to increase the permeability of the cytoplasmic membrane of *Escherichia coli* demonstrated its involvement in the first line of defence against bacterial invasion. Finally, histones were highly represented in zebra mussel hemocytes (1,323 SCs) compared to plasma (58 SCs) (Table 1). Nikapitiya et al. [88] reviewed the role of histone in the immune responses of aquatic invertebrates. In addition to their role in chromatin structure, histones are upregulated during immune challenges and exert antimicrobial peptide activity against bacteria and parasites [89]. Moreover, the release of antimicrobial histones and DNA in plasma by hemocytes of the pacific oyster have been associated with extracellular trap used to counteract pathogen propagation. Thus, the presence of histones in the plasma of the zebra mussel could suggest that extracellular trap mechanisms may also exist in the immune defence of *D. polymorpha*.

Many important immune processes are controlled by the collaborative action of proteases and their inhibitors which may act either on endogenous proteins, by cleaving their regulatory subunits and permitting their biological activity, or by causing the inactivation and degradation of proteins produced by microorganisms [33]. Proteases and protease inhibitors known to be involved in the immune system of aquatic invertebrates have been identified in the hemolymph of zebra mussels. Among proteases, numerous

cathepsins were observed in both compartments of the hemolymph, four times more abundant in hemocytes than in plasma (Table 1). In bivalves, the endopeptidase cathepsins have been identified in the razor clam (*Sinonovacula constricta*), the pearl oyster (*Pinctada fucata*) and the Pacific oyster [90–92]. While the implication of cathepsins in vertebrate immunity is well documented, its role within the innate immune defense of invertebrates is still poorly understood. However, recent investigations showed that gene expression of cathepsins was enhanced after bacterial and viral challenges in the razor clam and the freshwater prawn (*Macrobrachium rosenbergii*), suggesting its implication in the lysis of micro-organisms [91,93]. In vertebrates, cathepsins are involved in the regulation of defence activity and served as a chemical barrier against microbial invasion [94]. Protease inhibitors are used by bivalves to counteract the action of proteases produced by microorganisms [33]. Three main types of protease inhibitors potentially involved in the immunity of *D. polymorpha* were identified. While serine protease inhibitors were identified exclusively in hemocytes, cystatins and alpha-2-macroglobulins were found in both hemocytes and plasma (Table 1). In the hemolymph of the eastern oyster *C. virginica*, serine protease inhibitors have been implicated in the resistance against the parasite *Perkinsus marinus* and showed an ability to inhibit the major extracellular protease secreted by this pathogen [95]. Cystatin B is a cysteine protease inhibitor, which targets predominantly the cathepsin L-type [96]. Like that of cathepsins, the role of cystatins in immunity is not well documented for invertebrates. However, Cystatin B is produced in responses to bacterial infections in the leech (*Theromyzon tessulatum*), the disk abalone (*Haliotis discus discus*) and the Manila clam (*Ruditapes philippinarum*) [97–99]. All authors showed that cystatin B was sensitive to pathological conditions and suggested its implication in the inactivation of proteases produced by invading pathogens. Alpha2-macroglobulin genes have been characterized in the hemocytes of many bivalves, such as the sea scallop (*Chlamys farreri*) and the pearl oyster (*Pinctada fucata*) [100,101]. This protein is designed as a conserved arm of the innate immune system [102]. In invertebrates, this protease inhibitor has been shown to bind and neutralize diverse virulent proteases introduced by microorganisms [103]. Moreover, Ponprateep et al. [104] suggested that alpha2-macroglobulin was able to modulate the pro-phenoloxydase system of the Pacific white shrimp, *Litopenaeus vannamei*.

3.3.5 The Complement system

The complement system is a complex molecular machinery of the highest importance in the recognition and elimination of pathogens. Complement system acts as a critical first-line responder of innate immunity, its main functions are to discriminate and eliminate pathogens and promote inflammatory responses [105]. While the complement system has been well characterized in vertebrates, recent investigations confirmed its existence in invertebrates [106–109]. Core component of the complement system (C3 and C2/factor B) and recognition molecules involved in the activation of the complement system have been

recently documented in the hemolymph of bivalve species using genomics and transcriptomics analysis [110–112]. Moreover, key components of the complement such as C1q domain containing protein, C3 component and complement factor B were modulated in the hemolymph and extrapalleal fluid of bivalves during infectious experiments [110,112–114].

Gerdol et al. [33] discussed on mechanisms of the complement system in bivalves, which is devoid of the membrane attack complex (MAC) and antigen-complexed immunoglobulins. In this system, authors hypothesized that the complex arsenal of soluble PRRs existing in the hemolymph of bivalves, including C1q domain containing protein, CLTs and FReDs may participate in the activation of the complement system. Then, it is not clear how their recognition signals would converge towards C3, since no clear homologue of the serine proteases involved in the cleavage of C3 component has been identified in bivalves [33]. According to Wang et al. [112], complement components of oyster probably function as opsonins to assist the phagocytosis of pathogens by hemocytes rather than direct lysis of the pathogens. However, pore-forming enzymes such as lysozymes and BPIs could replace the terminal pathway of the complement system, leading to destruction of microbial invaders [33].

Numerous proteins related to the complement system were identified in the hemolymph of the zebra mussel. Among them, 9,771 spectra were attributed to the complement component C3 in plasma, representing 16.45% of the proteome, which makes it the most abundant protein identified in plasma (Table 1). C3 component is known to play a pivotal role in the complement system and was considered as the “Swiss Army Knife” of innate immunity and host defence [115]. In addition to the complement C3, proteins containing C1q domains and complement factor B-like proteins were also highly represented in plasma compared to hemocytes (Table 1). Like in vertebrates, proteins containing C1q domains work as PRRs that recognize microorganisms and may trigger the activation of the complement pathways in bivalves [112]. Complement Factor B is a serine protease which is considered as an important regulator of the activation of the component system. Similarly to the complement factor B characterized in the clam *Ruditapes decussatus*, proteins annotated as the complement factor B in the hemolymph of *D. polymorpha* were composed of a serine protease domain and complement control protein modules [107]. In total, proteins related to the complement system represent more than 20% of the plasma proteome (Table1). This suggests a strong involvement of the complement in the first line defence against invaders. However, the mechanisms of action of this process remain uncertain and require further analysis.

3.3.6 Proteins involved in apoptotic process

The establishment and functioning of the immune system involve apoptosis processes, which actively contribute to the immune system homeostasis. Well-characterized and conserved in vertebrates and invertebrates, this system involves two distinct signalling and execution pathways (intrinsic and extrinsic pathways) that lead to cellular suicide to support the immune system [24]. In molluscs, many studies have highlighted the essential role of hemocyte apoptosis in the immune defence against pathogens, protozoan parasites and environmental pollutants such as herbicides, insecticides and pharmaceutical products. Regulators and effectors of apoptosis were detected mainly in the hemocytes (590 SCs) of *Dreissena polymorpha* compared to plasma (114 SCs) (Table 1). Among them, cytochrome-c (cyto-c), apoptosis regulator BAX, apoptosis inducing factor (AIF) and several inhibitors of apoptosis (IAPs) were identified in the hemocyte compartments. These proteins are well known to be involved in the intrinsic mitochondrial apoptosis pathway. Briefly pro-apoptotic (Bax) and anti-apoptotic (IAP) members of the Bcl-2 family proteins control the mitochondrial membrane permeability [116]. An increase in mitochondrial membrane permeability generates the release of mitochondrial components and proteins into the cytoplasm (cyto-c and AIF) which contributes to the formation of an apoptosome complex (caspase 9/ cyto-c) that activates caspases and triggers apoptosis [116]. Caspase proteins were also identified mainly in hemocytes except for the caspase 3 which was identified in both compartments. According to the literature, this latter is commonly described in intracellular compartments. Thus, caspase 3 may end up in the plasma due to cell lyses caused by the centrifugation of hemocytes. Caspases are a class of cysteine proteases which participate in the initiation and the execution of intrinsic and extrinsic apoptosis pathways [116]. Depending on the mechanism of activation and their place in the cascade of events, caspases are divided into two subgroups. In the hemocytes of *D. polymorpha*, initiating caspases (8) and effective caspases (3 and 7) were detected by proteogenomics. Other *D. polymorpha* proteins involved in apoptosis were related to MAPKs and NF- κ B immune signalling pathways which are involved in the regulation of both intrinsic and extrinsic apoptosis pathways [116].

4. Conclusion

Next-generation proteomics is an interesting tool for a better understanding of the physiological processes of non-model organisms like *D. polymorpha*. In the present study, we applied state-of-the-art proteogenomics to document a large set of proteins from the hemolymph of the zebra mussel. We established the largest repertoire of proteins (3,020 identified by tandem mass spectrometry with at least two peptide sequences) for this not-yet genome sequenced sentinel organism. While several proteins were shared between plasma and hemocyte compartments, many of them were detected exclusively in the plasma, suggesting that proteins of plasma could have different origins. The GO analysis performed on immune-related proteins revealed that these two compartments exert closely

related immune functions, with few differences. Regarding the immune proteins of hemocytes and plasma, many proteins involved in recognition, internalization and elimination of microorganisms were identified, which underlined the importance of both compartments in the management of foreign particles. The proteogenomics analysis has provided access to identify immune proteins rarely observed in the hemolymph of bivalves. Indeed, a natterin-like protein was observed in high abundance in hemocytes and plasma. While this protein is known to be involved in the recognition and elimination of microorganisms in fish species, its role in bivalve immunity remains to be investigated. Like natterin, a thaumatin-like protein was abundant in the hemolymph of *D. polymorpha* and its role in the immune defence of bivalves is still poorly studied and need further investigations. Furthermore, proteogenomic analysis of the plasma compartment revealed new insights into immune mechanisms occurring in the plasma of bivalves. For example, a large amount of complement-related proteins observed in plasma could confirm the existence of a complement system in bivalves and suggest a strong implication of this mechanism in the management of invaders.

Considering this study, dissolved immune proteins from the plasma compartment work in a complementary fashion to hemocytes to neutralize invading organisms and manage immune responses of *D. polymorpha*. Transcriptomic analysis of the hemolymph only focusses on the modulation of hemocyte genes and does not take into consideration complex post-transcriptional regulation and post-translational modifications of polypeptides that occur at large scale in animals. Our proteogenomics strategy proved its capacity to map and quantify the immune proteins in both compartments. Taking into account the possible immune interactions between hemocytes and plasma, such analysis provides a better understanding of immune responses. Thus, we recommend that future research on bivalve immunology should take into consideration the molecular responses from the whole hemolymphatic tissue, including both hemocyte and plasma compartments. Further investigations with targeted quantitative proteomics are now needed to characterize the dynamic of immune proteins during stressful conditions and validate their implications in the management of contaminants [117,118]. This preliminary study paves the way for future proteomics investigations on zebra mussel immunity. This study contributes to the understanding of immune responses of *D. polymorpha* and the discovery of novel molecular immuno-markers that could be used in aquatic ecotoxicology.

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Table 1. Abundance of Immune proteins identified in hemocytes and plasma compartments

1194

1195

1196 **Appendix A. Supplement Material**

1197 **Supplement Table S1. Identified proteins from the hemocytes.**

1198 Protein scores, percent peptide coverage of protein, spectral count of biological replicates,
1199 NSAF values, functional annotations and GO-term assignment are displayed for each protein.

1200

1201 **Supplement Table S2. Identified proteins from the plasma.**

1202 Protein scores, percent peptide coverage of protein, spectral count of biological replicates,
1203 NSAF values, functional annotations and GO-term assignment are displayed for each protein.

1204

1205 **Supplement Table S3. Conserved domain search of proteins from the hemolymph.**

1206 **Supplement Table S4. List of peptides identified from the hemocytes.**

1207 Peptide sequences, peptide scores and peptide modifications are displayed for each protein
1208 and each sample of hemocytes.

1209

1210 **Supplement Table S5. List of peptides identified from the plasma.**

1211 Peptide sequences, peptide scores and peptide modifications are displayed for each protein
1212 and each sample of plasma

1213

1214 **Conflicts of Interest**

1215 The authors declare no conflict of interest.

1216

1217 **Abbreviations**

1218 MAMP : Microbe-associated molecular pattern
1219 DAMP : Damage-associated molecular pattern
1220 RNA-seq : mRNA sequencing
1221 MS/MS: Tandem mass spectrometry
1222 NSAF : Normalized spectral abundance factor
1223 SC : Spectral count
1224 NCBI : National Center for Biotechnology Information
1225 GO : Gene Ontology
1226 PSM : Peptide spectrum matches
1227 Db : Data base
1228 PRR : Pattern recognition receptors
1229 CTLs : C-type lectins
1230 FREPs : Fibrinogen-related proteins
1231 FrD : Fibrinogen-related domain
1232 MPR : Mannose-6-phosphate receptor
1233 GBP : Beta-1,3-glucan-binding protein
1234 PGRP : Peptidoglycan-recognition protein
1235 AIF-1 : Allograft inflammatory factor 1
1236 MIF : Macrophage migration inhibitory factor
1237 MAPK : Mitogen-Activated Protein Kinase

1238 JNK : c-jun N-terminal kinase
1239 ERKs : Extracellular signal-regulated kinases
1240 IFN : Interferon
1241 STAT : Signal transducers and activators of transcription
1242 NF- κ B : Nuclear factor kappa B
1243 PKC : Protein kinase C -type
1244 PI3K : Phosphatidylinositol 4,5-bisphosphate 3-kinase
1245 Arp2/3 : Actin-related protein 2/3
1246 CDC42 : Cell Division Cycle 42
1247 Rac1 : Ras-related C3 botulinum toxin substrate
1248 NWASP : Neural Wiskott-Aldrich syndrome protein
1249 AP : Adaptor protein
1250 AMP : Antimicrobial peptide
1251 TLP : Thaumatin-like protein
1252 BPI : Bactericidal/permeability-increasing protein
1253 cyto-c : Cytochrome-c
1254 AIF : Apoptosis Inducing Factor
1255 IAP : Inhibitor of apoptosis

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Table 1. Abundance of Immune proteins identified in hemocytes and plasma compartments

Protein functions	Hemocytes		Plasma	
	Spectral counts	% NSAF	Spectral counts	% NSAF
Recognition of micro-organisms				
Natterin-like protein	804	2.19	898	2.69
Galectins	588	1.96	484	1.41
MytiLec-like proteins	185	0.86	171	0.94
Fibrinogen-related proteins	222	0.32	428	0.74
Beta-1,3-glucan-binding proteins (BGBP)	269	0.17	203	0.13
C-type lectins	169	0.14	956	1.34
Peptidoglycan-recognition proteins	36	0.05	378	0.53
Cation-dependent mannose-6-phosphate receptors	-	-	57	0.01
Protein involved in immune signalling				
Mitogen-activated protein kinases	320	0.21	80	0.05
Proinflammatory cytokines	84	0.10	43	0.06
Interferon-Responsive Factors	97	0.06	-	-
PKC and PI3k related proteins	77	0.03	80	0.21
Nuclear factor kappa B pathway	37	0.01	-	-
Internalization of microorganisms				
GTPases of the Rho family	1890	1.16	325	0.24
Actin-related protein 2/3 complex	1017	0.77	309	0.23
Integrin related proteins	941	0.57	85	0.05
Clathrin-mediated endocytosis	349	0.10	37	0.01
Antimicrobial peptides				
Defensin	13	0.04	-	-
Thaumatins	201	0.23	149	0.18
lysozymes	7	0.01	51	0.08
Histones	1323	2.64	58	0.14
Proteases and Protease Inhibitors				
Cathepsins	455	1.32	110	0.29
Other proteases	363	0.19	1251	1.53
SERine Proteinase INhibitors (serpins)	31	0.04	-	-
Alpha-2-macroglobulins	756	0.47	4468	4.24
Cystatins	238	0.99	162	0.71
Complement system				
Complement component C3	585	0.96	9771	16.45
c1q domain containing proteins	194	0.68	1371	2.12
Complement component C2/factor B	72	0.17	1392	3.11
Other proteins related to complement system	12	0.05	310	1.16
Apoptosis				
Apoptosis regulators	460	0.27	86	0.06
Caspases	130	0.09	28	0.03

Non model species

Zebra mussel

Nano LC-MS/MS

Hemolymph proteome

Proteogenomic

RNA sequencing